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Influence of Viraemia and Genotype Upon Serological Reactivity in Screening Assays for Antibody to Hepatitis C Virus

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Detection of antibody to recombinant proteins derived from hepatitis C virus (HCV) genotype 1 represents the principal method for diagnosis of HCV infection. A method was developed for quantifying antibody reactivity in two third-generation enzyme immunoassays (Ortho EIA 3.0 and Murex VK48), and the influence of viraemia, HCV genotype, and host factors such as age, gender, and risk group upon antibody levels were investigated in a consecutive series of 117 anti-HCV-positive volunteer blood donors. Viraemic donors (as assessed by the polymerase chain reaction; PCR) showed significantly higher levels of anti-HCV by the Ortho EIA than those who were nonviraemic (adjusted mean difference of 10.1 fold after multiple regression analysis). The only other factor to influence significantly antibody level was genotype, where it was found that donors infected with type 1 showed 4 to 4.5 times greater serological reactivity by the Ortho assay than those infected with type 2 or 3. Antibody levels by the Ortho assay correlated closely to those detected by the Murex VK48 assay, and similar differences between PCR-positive and negative donors and between those infected with different genotypes were found. Differences in serological reactivity between genotypes indicate that a large proportion of epitopes of the type 1a or 1b recombinant proteins used in current assays are genotype specific. Variation in sensitivity of screening assays for different genotypes is of potential concern when used in countries where non-type 1 genotypes predominate in the blood donor or patient population.

KEY WORDS: blood donor, screening, ALT, antigenic variation

INTRODUCTION

Following the identification of hepatitis C virus (HCV) as the principal aetiological agent of posttransfusion non-A, non-B hepatitis [Kuo et al., 1989; Choo et al., 1989], many countries have established routine screening of all blood donors for anti-HCV-specific antibody by enzyme immunoassay (EIA). Most commonly used assays use recombinant proteins derived from the prototype HCV clone, HCV-PT (genotype 1a). However, other variants of HCV may differ substantially in nucleotide sequence from one another and show varied geographical and epidemiological distributions [Bukh and Miller, 1994; Simmonds, 1995]. In particular, the inferred amino acid sequences of the envelope glycoproteins and nonstructural proteins differ considerably, leading to the possible existence of type-specific as well as shared epitopes between genotypes. It remains unclear whether such assays are equally effective for detecting antibody elicited by heterologous genotypes.

Previous investigations of serological reactivity to individual HCV proteins by immunoblotting assays indicated type-specific reactivity to nonstructural proteins used as antigens in current screening enzyme-linked immunosorbent assays (ELISAs). For example, reactivity to c100-3 and 5-1-1 antigens used in the original first-generation screening assays for HCV was found more frequently in blood donors infected with HCV type 1a or 1b (90%) than in those infected with type 2 or 3 [30–35%; Chan et al., 1992; McOmish et al., 1993]. Although second and third-generation screening assays for antibody to HCV contain a wider range of antigens, the frequency and strength of serological reactivity to each component have also been shown to vary between genotypes. For example, individuals in-

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Study was undertaken at University of Edinburgh.

fected with type 3 were statistically more likely to show indeterminate results by second-generation recombinant immunoblot assay (RIBA-2; Chiron, Emeryville, CA) generally reacting with only the highly conserved c22-3 protein (90% amino acid sequence similarity between genotypes), and not to the more variable nonstructural proteins NS-3 and NS-4 [McOmish et al., 1994].

Although differences in serological reactivity exist between HCV genotypes, it has proved extremely difficult to obtain direct information on whether this is sufficient to reduce significantly the effectiveness of current screening assays for anti-HCV used for blood donor screening. This is partly because analyses of serological reactivity have so far been confined to samples that have already been identified as anti-HCV positive. In addition, no practical antibody assay has been produced that is based upon a different HCV genotype so preventing a comparison with conventional assays. Other methods of detection of infection that are independent of serology, such as the polymerase chain reaction (PCR), are impractical for use on large numbers of blood donor samples.

In the current study a method was developed to quantify levels of antibody to HCV antigens using two commercial assays (Ortho EIA 3.0 [Ortho Diagnostic Systems, Raritan, NJ] and Murex VK48 [Murex, Dartford, Kent, U.K.] currently employed for blood donor screening amongst blood donors infected with different genotypes. Both assays are third generation and contain antigens corresponding to regions within the core, NS-3, NS-4, and NS-5 proteins encoded by viruses of genotype 1a (Ortho) or 1b (Murex). Quantitation of antibody levels allowed a detailed analysis of the factors that influenced serological reactivity to genotype 1 recombinant proteins, including viraemia status, genotype, and donor factors such as age, risk factors for infection, and gender.

MATERIALS AND METHODS

Donor Samples

Aliquots of plasma were obtained from consecutive Scottish blood donors who were repeatedly reactive by second or third-generation anti-HCV screening using Abbott, Ortho, or Murex assays. A total of 117 study samples were selected for the study according to the following criteria: a) Samples were either confirmed by RIBA-2 (n = 108), or were RIBA-2 indeterminate but PCR positive (n = 9; all type 3). b) For each sample used, it was possible to recall the blood donor and obtain a range of baseline data, such as age, gender, risk factor(s) for infection, alanine aminotransferase level, and geographical origin.

HCV Genotyping

HCV genotypes were determined by a serological typing assay based upon the detection of antibody to type-specific epitopes in the NS-4 region [Bhattacherjee et al., 1995]. This assay shows high sensitivity and excellent concordance with genotyping assays such as restriction fragment length polymorphism (RFLP) assays in this blood donor population and in patients with chronic hepatitis [Lau et al., 1995; Bhattacherjee et al., 1995]. The majority of samples used in the current study had been the subject of a previous comparison with an RFLP genotyping assay, and showed concordant results in 121 of the 122 samples that could be tested by both methods [Simmonds et al., 1993]. The single discrepant sample (type 3 by RFLP, type 1 by the serological genotyping assay) was not used in this study. The advantage of the serotyping assay was its ability to determine genotype in samples that were PCR negative (22 of the 116 donations described here; Table I), although in these there can by definition be no corroborating evidence from genotyping assays that the genotypes were correctly identified. However, inclusion of these results allowed the contributions of viraemia and genotype to be assessed simultaneously by multiple regression analysis (see below).

TABLE I. Genotypes, Viraemia Status, and Background of the Study Group

	HCV genotype[a]			
	1	2	3	Total[b]
Total	50	15	52	117
Viraemia status				
PCR+	37	13	44	94
PCR−	13	2	8	23
Gender				
Male	27	10	34	71
Female	20	2	15	37
Risk group				
IVDU	20	3	21	44
Other[c]	16	6	15	37
Unknown	11	4	8	23
Age range[d]				
20–29	20	1	18	39
30–39	17	3	20	40
40–49	6	7	5	18

[a]Genotype determined by serotyping assay [Bhattacherjee et al., 1995].
[b]Background information was not available from a small, variable proportion of donors, so that this column does not always add up to 117.
[c]This category includes previous transfusion (n = 17); tattooing/other parenteral exposure (n = 9), IVDU contact (n = 10), and family contact (n = 1).
[d]Treated as a continuous variable by multivariate analysis.

Viraemia was assessed by PCR as described previously [Chan et al., 1992]. 0.5 ml aliquots of plasma was ultracentrifuged at 100,000g for 2 hours at 4°C and RNA was extracted from the pellet by incubation in proteinase K/Sarkosyl prior to phenol/chloroform extraction, ethanol precipitation, and resuspension in 20 μl nuclease-free water. Five microlitres of RNA was reverse transcribed using an antisense primer (209) specific for the 5′ noncoding region (5′NCR), followed by amplification using primers 209 and 939 for the first round and 211 and 940 for the second, nested PCR. This method has an overall sensitivity of 200 copies of RNA/ml.

Antibody to individual antigens c22-3, c33c, c100-3, and 5-1-1 was assayed using RIBA-2 (Chiron) in accordance with the manufacturer's instructions.

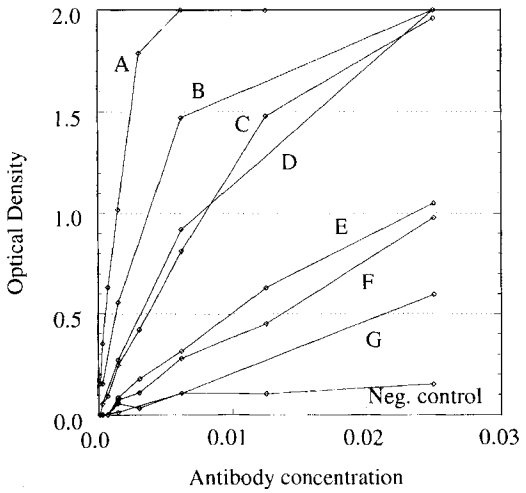

Fig. 1. O.D.s of anti-HCV-positive plasma samples (A-G) and an anti-HCV-negative control assayed at different dilutions in the Murex VK48 EIA. Sample dilutions expressed as concentration (e.g., a 1/40 dilution corresponds to a concentration of 0.025, 1/160 to 0.00625).

Measurement of Antibody Levels

Quantitation of antibody by the Ortho third-generation EIA (Ortho 3.0 EIA) and Murex VK48 EIA was carried out by assay of multiple dilutions (in anti-HCV-negative plasma) of samples as described in Results. In all other respects, each EIA was carried out in exact accordance with the manufacturer's instructions. To maintain consistency, the same anti-HCV-negative plasma sample was used for all dilutions. The positive control was another large volume plasma sample from an HCV type 1a-infected individual that was assigned a nominal antibody concentration of 1.0 unit, and which was used in all assay runs.

Because of the linear relationship between optical density (O.D.) and concentration of sample added to each well (over the range from 0 to 1.0; Fig. 1), anti-HCV reactivity could be quantified in terms of O.D., provided each sample was diluted sufficiently to produce an O.D. in the linear range of the assay. Each EIA plate included replicate dilution series of the reference sample, providing at least four absorbance values (two at each dilution) within the linear range of the assay. Antibody levels in test samples were measured relative to the mean value obtained from the reference sample on the plate on which they were tested. This controlled for the observed minor variation between plates and between batches in the performance of the EIA (±10–20% in absorbance values). For calculation of antibody levels, the O.D. value obtained from the negative control (also used as diluent for the test and reference specimens), representing nonspecific binding to the wells, was subtracted from both test and reference O.D.s. Insufficient volumes were available to measure antibody levels for one sample in the Murex VK48 assay and for seven samples in the Ortho EIA 3.0.

This method was chosen over titration to an end-point as it has advantages of accuracy, and controls for the effect of plate-to-plate variation. However, because of the linear relationship between concentration of sample added and absorbance, it would be possible in principle to convert each antibody level measured in this study to a dilution at which an O.D. assigned as the cut-off value (e.g., 0.1) would be obtained. This would allow results to be expressed as end-point titres. An analysis of several different methods for antibody quantitation has been presented elsewhere [Simmonds, 1987].

RESULTS

Measurement of Antibody Levels

Over a wide range of values, O.D.s by the Murex screening assay were proportional to the concentration of anti-HCV-positive plasma added (Fig. 1). Although the concentration of antibody within each specimen varied, a linear relationship was found between O.D. values of 0 to 1.0. For the following calculations we have assigned sample C as the reference antiserum with a nominal antibody level of 1.0 unit. Antibody levels in other samples relative to C can be calculated using the following formula:

$$\text{Test antibody level} = \frac{\text{Test O.D.} \times \text{reference concentration}}{\text{Ref. O.D.} \times \text{test concentration}}.$$

For example the O.D. of sample E is 0.632 at a concentration of 0.0125 (dilution of 1/80), while the reference sample has an O.D. of 0.812 at 0.00625. Therefore, sample E has a level of $(0.632 \times 0.00625)/(0.812 \times 0.0125) = 0.39$ arbitrary units of antibody (or about 4/10 the level in C). Because of the proportionality between O.D. and antibody concentration, essentially the same results can be obtained by selecting different test or reference dilutions. For example, the antibody level of sample E was 0.38 using the O.D. at a concentration of 0.00625 (O.D. = 0.315). Using the above formula the amounts of anti-HCV antibody for the samples are as follows: A) 3.1 units; B) 1.4 units; C) 1.0 units; D) 0.67 units; E) 0.38 units; F) 0.28 units; G) 0.18 units. A similar linear relationship between antibody concentration and O.D. was observed upon titration of the same samples in the Ortho EIA 3.0 (data not shown).

Clearly, the most accurate results are obtained using dilutions of antibody that produce O.D.s of at least 0.2 and less than 1.0, beyond which point the linear relationship with antibody amount was lost (Fig. 1). These limits were applied to antibody level measurements for all samples by both assays in this study.

Study Group

Approximately 80% of samples from the study group were HCV RNA PCR positive and the frequency of viraemia was similar amongst the three HCV genotypes detected in this population (74% type 1; 87% type 2; 85% type 3). Genotype distributions varied in male and

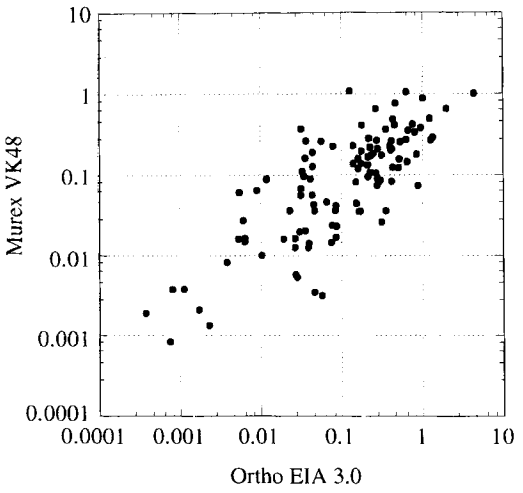

Fig. 2. Comparison of antibody levels in the Ortho EIA 3.0 and Murex VK48 EIAs for the study samples, plotted on a $\log_{10}$ scale.

female donors (males accounted for 57%; 83%; 70% of infections with types 1, 2 and 3 respectively), and there was under-representation of type 2 amongst donors in whom drug abuse was identified as the principal risk factor (3/13 for type 2 compared with 20/47 and 21/23 for types 1 and 3). Possibly related to this observation is the marked difference in age distribution between type 2 infected donors (median 41 years) compared with type 1 (30 years) and type 3 (30 years).

Antibody Levels in Blood Donor Samples

Each of the study samples was assayed by both Ortho and Murex screening EIAs at a series of dilutions (usually 1/40, 1/160, and 1/640) to measure antibody level relative to the reference sample. Values ranged from 0.0007 to 3.5 in the Ortho assay (a 5,000-fold range), and from 0.0008 to 1.02 in the Murex assay (range 1,275-fold). A close correlation was found between antibody levels measured in the two assays (Fig. 2), which was highly significant by regression analysis ($r = 0.787$; $P = 0.0001$).

Analysis of Factors Influencing Antibody Levels

The levels of circulating antibody detected by the two screening assays could be influenced conceivably by a wide range of virus and host-specific factors. These include viraemia, HCV genotype (see Introduction), route of infection/risk group, donor age, and gender. With the Ortho assay, the distribution of antibody levels differed between samples that were PCR positive and negative (Fig. 3A) and also between genotypes (Fig. 3B). There was a 20-fold difference in the median antibody level for PCR-positive samples (0.215), compared with PCR negatives (0.010), and a similar difference was found for the Murex VK48 assay (0.126 compared with 0.013; Fig. 3E). A similar difference in antibody levels by both assays was also detected between different genotypes (Fig. 3A,D). In the Ortho assay, median antibody levels were approximately fivefold higher in type 1-infected donors compared with those infected with type 3 (medians 0.275 and 0.056, respectively), similar to the difference observed using the Murex assay (medians of 0.185 and 0.065).

There was no significant differences in antibody levels by either assay between males and females (data not shown), age (Fig. 3C,F), or risk group (data not shown). Thus, there was no evidence from this preliminary analysis that these host factors could account for the observed differences in antibody level between genotypes or between PCR positives and negatives.

In order to investigate the relative contribution of viraemia and genotype to antibody levels, and the extent to which these are influenced by other factors, we carried out multiple regression analyses of both assays fitting genotype, risk group, gender, and age as covariates. This allowed the size and significance of differences between groups to be calculated and controlled by other factors.

By this analysis, viraemia and genotype proved to be the principal influences upon antibody level by both assays. Adjusted antibody levels by the Ortho assay were 10.2 times higher in viraemic donors, and between 4 and 4.5 times higher in type 1-infected donors compared with genotypes 2 and 3. Similar although less marked differences between these categories emerged from comparison of antibody levels by the Murex VK48 assay. The only other comparison that approached significance was the observed higher levels of antibody by the Murex assay in intravenous drug abusers (IVDUs) compared with donors infected by other parenteral routes. No difference, however, was found by the Ortho assay. Age was not correlated with antibody level in either assay ($P = 0.52$, $P = 0.33$, respectively; n.s.).

Alanine Aminotransferase (ALT) Levels

Although antibody levels were the principal outcomes investigated in this study, ALT levels were investigated as an alternative outcome of these host factors. Multiple regression analyses of ALT fitting genotype, risk group, gender, and age as covariates were carried out. The only significant influence upon ALT levels was viraemia, where PCR-positive donors showed an adjusted mean ALT level approximately 20 times higher than PCR negatives. With adjusted data there was no evidence for a difference in ALT levels between genotypes, risk group, gender, or age (Table II; age: $P = 0.70$).

DISCUSSION

The main influences upon anti-HCV antibody level as measured by either Ortho or Murex assays in this study were viraemia and genotype, with significantly higher levels being found in donors who were PCR positive and infected with genotype 1. The detection of circulating virus by PCR indicates the presence of active HCV infection, and the higher antibody levels observed in this group may result from persistent stimulation of the immune system. This explanation is consistent with the finding of an association between

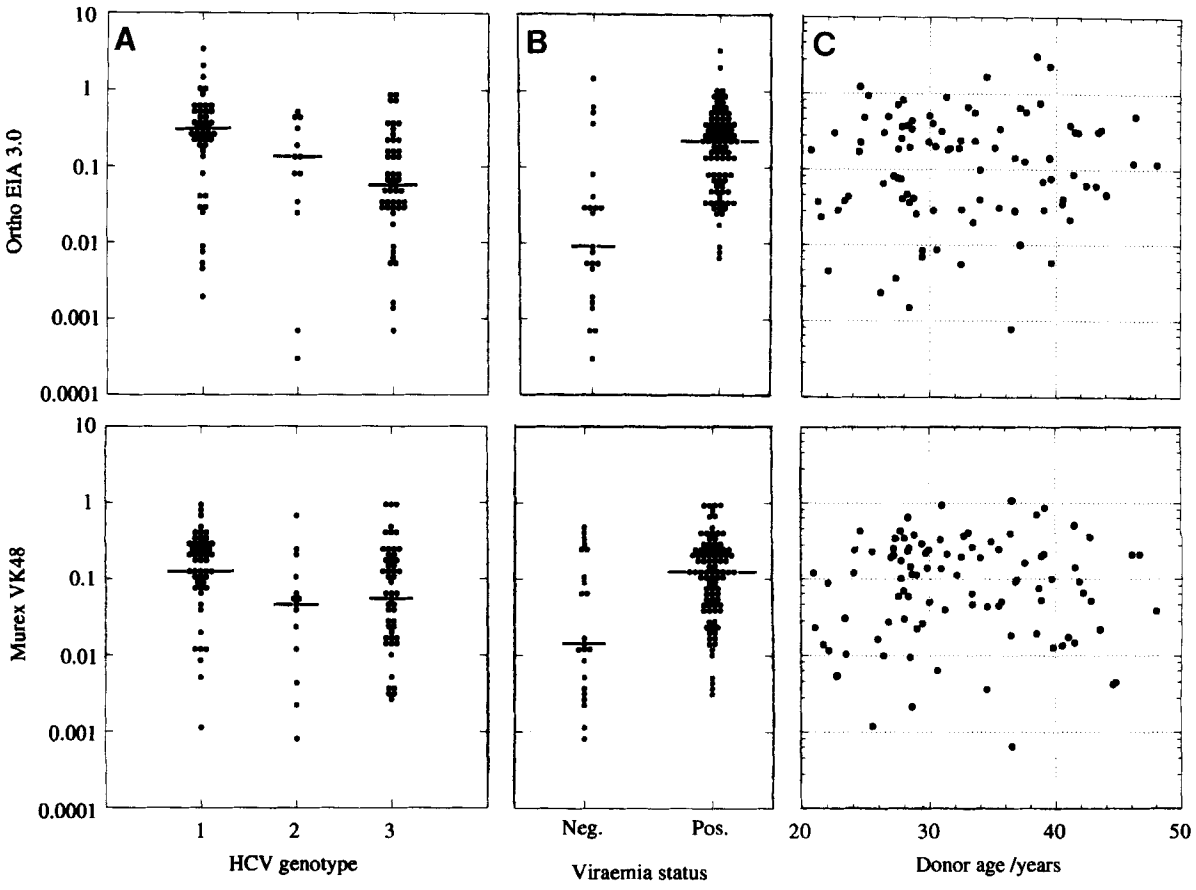

Fig. 3. Distribution of antibody levels in Ortho EIA 3.0 (**A, B, C**) and Murex VK48 EIA (**D, E, F**) in study group categorised for viraemia status (A, D), genotype (B, E), and age (C, F). Median values indicated by horizontal bar.

TABLE II. Adjusted Influence of Viraemia, Genotype, and Host Factors Upon a Antibody and ALT Levels

	Ortho EIA 3.0		Murex VK48		ALT	
Factor[a]	Adjusted ratio[b]	*P* value*	Adjusted ratio	*P* value*	Adjusted ratio	*P* value*
Viraemia						
Positive-negative	10.2	<0.00001	3.54	0.0011	0.053	0.032
Genotype						
Type 1-type 2	4.12	0.005	3.72	0.014	0.97	0.95 (n.s.)
Type 1-type 3	4.48	<0.00001	2.35	0.0070	0.84	0.47 (n.s.)
Type 3-type 2	0.93	0.88 (n.s.)	1.58	0.38 (n.s.)	1.16	0.76 (n.s.)
Gender						
Female/male	1.06	0.83 (n.s.)	1.63	0.13 (n.s.)	0.68	0.12 (n.s.)
Risk						
IVDU-other	1.22	0.52 (n.s.)	2.31	0.049	0.84	0.50 (n.s.)
IVDU-unknown	1.24	0.59 (n.s.)	1.81	0.075 (n.s.)	1.03	0.93 (n.s.)
Other-unknown	1.01	0.97 (n.s.)	1.28	0.57 (n.s.)	1.23	0.55 (n.s.)

[a]Age is a continuous variable and cannot be represented in the table (see text).
[b]Adjusted ratio of antibody or ALT levels after multiple regression analysis between the first and second category (e.g., PCR positive compared with PCR negative).
*Significant *P* values (<0.05) are underlined; nonsignificant values indicated (n.s.).

viraemia with ALT abnormalities, presumably reflecting a link between virus replication and liver damage. Amongst the PCR-negative group, most had ALT levels within the normal range for blood donors (0-55 IU/ml), suggesting low levels of virus replication or complete clearance of HCV.

In this study we sought to investigate the extent to which antigenic variation between genotypes affects

the sensitivity of current screening assays. Lower serological reactivity was found to the type 1a or 1b antigens used in the EIAs with samples from donors infected with non-type 1 genotypes. The study was designed to examine the separate influences of host factors that might compound the observed differences between genotypes in serological reactivity. By multivariate analysis, no evidence was found for any significant effect of age, risk group, or gender upon antibody reactivity, while the genotype differences were shown to be independent of the differences in antibody levels between PCR-positive and -negative samples (see above). The magnitude of the difference in levels provides an estimate of the relative amounts of genotype-specific and cross-reactivity antibody to the core, NS-3, NS-4, and NS-5 antigens. For example, the 4-4.5-fold difference between type 1 with types 2 and 3-infected donors could be interpreted as indicating that a major proportion of antigenic determinants in the EIAs are genotype specific. This conclusion is consistent with the finding of type-specific epitopes in core, NS-4, and NS-5 regions [Machida et al., 1992; Simmonds et al., 1993; Tanaka et al., 1994; Bhattacherjee et al., 1995; Zhang et al., 1995]. Although detailed mapping of type-specific epitopes in the NS-3 and NS-5 regions used in screening assays has not been published, the existence of significant amino acid sequence variability in both of these regions predicts the existence of substantial antigenic variation that may also contribute to the observed differences in this study.

This analysis cannot rule out completely the possibility that type 1 infections elicit higher levels of antibody than other genotypes, although if this were the case then it would be expected that type 1 infections would be associated with a higher mean ALT level if antibody level was related to a greater severity of disease. This is clearly not the case, as it was found that adjusted mean ALT values were within 16% of each other (Table II). Similarly, although there is abundant evidence that type 1-infected patients are less likely to respond to interferon treatment than types 2 and 3, evidence for a difference in pathogenicity between genotypes has proved more elusive. A similar spectrum of liver disease has been described in patients infected with each of the genotypes, with little evidence that type 1 is more pathogenic once the effect of age (and duration of infection) is taken into account. Recently, it has been shown that mean levels of viraemia are similar between genotypes 1, 2, and 3, once the greater sensitivity of the bDNA assay for type 1 sequences is taken into account [Smith et al., in press; Lau et al., submitted], effectively discounting any inherent difference in replicative capacity or immunological control between them in vivo. Consistent with these new data is the previous observation that multiple-exposed individuals such as haemophiliacs do not show an overrepresentation of any particular genotype compared with the distribution of genotypes in the plasma from which the blood products were manufactured [Jarvis et al., 1994], indicating that type 1 in particular shows no tendency to replace other genotypes upon reinfection.

This study provides grounds for concern that antigenic variability of HCV impairs the performance of current assays. For example, if the current assays were 4.5 times less sensitive than they currently are (the difference between antibody levels of type 1 and 3 donors; Table II), then it is possible that a proportion of samples currently detected by screening assays would become negative. We have retested samples from the 50 type 1-infected donors in the Ortho EIA at a 1/90 dilution compared with the recommended 1/20 dilution (therefore making the assays equivalent to their current sensitivity for type 3-infected donors), and observed a decrease in O.D.s for many of the samples. Of interest was the observation that one RIBA-confirmed sample became borderline at the 1/90 dilution (O.D. 0.69; assay cut-off 0.65). Further studies are required to investigate the clinical significance of these differences in sensitivity.

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